Regulation of Atg1 Protein Kinase Activity by Phosphorylation in *Saccharomyces cerevisiae*

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for graduation *with research distinction* in Molecular Genetics in the undergraduate colleges of The Ohio State University

By

Kristie Wrasman

The Ohio State University
May 2010

Project Advisor: Professor Paul K. Herman, Department of Molecular Genetics

Abstract

Autophagy is an evolutionarily conserved pathway that is responsible for the turnover of non-essential proteins and organelles during periods of nutrient limitation or stress. The products of this process are recycled and used for the synthesis of the macromolecules needed for survival. The autophagy process is being considered as a point of therapeutic intervention in many diseases including many cancers and neurodegenerative disorders, like Huntington's disease. It is therefore critical that we develop a thorough understanding of autophagy regulation. Toward this end, my work has focused on the Atg1 protein kinase, a key point of regulatory control within this pathway in all eukaryotes. Atg1 is targeted by a number of signaling pathways that are responsible for coordinating growth with nutrient availability. My studies were focused on developing a better understanding of how these pathways influence autophagy by controlling the phosphorylation status of Atg1. In particular, we used mass spectrometry (MS) to identify candidate phosphorylation sites on Atg1 and then assessed the physiological significance of these sites for autophagy in *Saccharomyces cerevisiae*. Site-directed mutagenesis was used to individually replace different serine (or threonine) residues that were normally phosphorylated with either a nonphosphorylatable alanine or a phosphomimetic (e.g. glutamic acid). These studies identified Thr-226 phosphorylation within the Atg1 activation loop as a positive regulator of both Atg1 kinase activity and autophagy. A number of other mutants analyzed demonstrated a modest decrease in autophagy. These sites may work cooperatively to regulate kinase activity and autophagy.

Acknowledgments

I would like to thank everyone in the lab that assisted me in my research during my two years in the lab especially Vidhya Ramachandran, and Khyati Shah. I would especially like to thank Yuh-Ying Yeh for helping me to discover my passion, and being the most patient teacher ever. A special thanks to Dr. Paul Herman for his time and giving me the opportunity to conduct research. Finally, I am very grateful to my parents for all their support and encouragement over the years.

Table of Contents

Chapter 1

Introduction

Autophagy is a highly conserved degradative pathway that non-specifically engulfs portions of the cytoplasm during periods of nutrient limitation (Tsukada and Ohsumi 1993). At the pre-autophagosomal structure (PAS), a double-membrane surrounds proteins and other components within the cytoplasm to form an autophagosome (Suzuki and Ohsumi 2007). This autophagosome is targeted to the lysosome in mammalian cells or the vacuole in yeasts (Xie and Klionsky 2007). The encapsulated macromolecules are then broken down into their constituent components, which are reused by the cell to allow for their survival during this period of stress.

Autophagy has been linked to a wide variety of human ailments, including cancers and neurodegenerative diseases. Depending on the type of cancer, autophagy has been found to either promote or inhibit tumor progression. For example, cancerous cells divide uncontrollably to form tumors even when there are not enough nutrients to sustain the aberrant growth. Autophagy helps to maintain these cancerous cells because they are able to reuse their own components to continue living before angiogenesis within the tumor (Levine and Kroemer, 2008). In contrast, autophagy may contribute to specific programmed cell deaths and the inactivation of autophagy has been implicated in the progression of specific cancers, including breast and ovarian cancers (Chen *et al.* 2009). Also, autophagy inhibits neurodegenerative disorders like Alzheimer's and Huntington's disease, which are associated with the accumulation of toxic aggregates of certain proteins in the neurons of the afflicted brains. Autophagy strives to recycle these aggregates so that they do not increase to toxic levels (Levine and Kroemer, 2008). Thus,

there has been tremendous interest in manipulating autophagy as a possible means of therapeutic intervention for these diseases. This objective in turn necessitates a more complete understanding of the activation and regulation of autophagy, the central focus of this work.

Autophagy has been conserved through evolution, from single-celled yeasts to humans. In fact, most of our knowledge of this process comes from work on *Saccharomyces cerevisiae,* bakers' yeast. In yeast, over 30 Atg proteins are involved in autophagy, and most of them have functional counterparts in human cells (Meijer *et al.* 2007; Tsukada and Ohsumi 1993). Atg1 is a serine/threonine-specific protein kinase, whose activity is required for the induction of autophagy. Atg1, and its associated proteins, form a complex that appears to be a key point of regulatory control for the autophagy process (Budovskaya *et al.* 2005; He and Klionsky 2009; Kamada *et al.* 2000; Stephan *et al.* 2009). In particular, both Atg13 and Atg17 are required for Atg1 kinase activity and are found in this complex necessary for the autophagy process (Kamada *et al.* 2000; Stephan *et al.* 2009).

Protein phosphorylation is a major post-translational modification used to regulate many biological processes, including the autophagy pathway. Recent studies have demonstrated that two major signaling pathways required for the proper control of *S. cerevisiae* growth directly target components within the Atg1 complex and thereby inhibit the autophagy process. The Tor protein kinase directly phosphorylates Atg13 and the cAMP-dependent protein kinase modifies both Atg1 and Atg13 *in vivo*. Each of these modifications has been shown to interfere with the assembly of the Atg1 complex in *S. cerevisiae* (Budocskaya *et al.* 2004; Kamada *et al.* 2010; Noda and Ohsumi 1998;

Stephan *et al.* 2009). I found that within the Atg1 kinase domain, Thr-226 is an important site of regulatory control. The autophosphorylation of Thr-226, within the activation loop, is necessary for Atg1 kinase activity and the induction of autophagy. Thr-226 is evolutionarily conserved in all Atg1 orthologs suggesting that this phosphorylation event may be an important point of regulation for this pathway in all eukaryotes. In addition, a recent mass spectrometry analysis of Atg1 performed in our lab has identified 15 additional sites of phosphorylation within this protein. Given the complex regulation of the autophagy pathway and the importance of Atg1 for this control, we set out to characterize the potential regulatory roles of these Atg1 phosphorylation events. Using site-directed mutageneses, we found that the alteration of most sites had only a modest effect upon the autophagy process. To regulate kinase activity and autophagy, multiple sites of phosphorylation may work cooperatively.

Chapter 2

Materials and Methods

Plasmid constructions:

Table 1

Plasmid	Genes
pPHY 1115	3xmyc-tagged Atg1 in pRS 316 generously provided by Dr. Ohsumi
pPHY 1249	3x myc-tagged Atg1 in pRS 424
pPHY 2622	Atg1 with T226E mutation in pRS 316, previously made from pPHY 1115
pPHY 2624	Atg1 with T226A mutation in pRS 316, previously made from pPHY 1115
pPHY 2792	Atg1 with S_{621}A mutation in pRS 424, previously made from pPHY 1249
pPHY 2793	Atg1 with S_{677}A mutation in pRS 426, previously made from pPHY 1249
pPHY 3514	Atg1 with S677A, and S683A mutations in pRS 316, previously made from pPHY 1115
pPHY 3521	Atg1 with S_{621}A, S_{677}A, S_{683}A mutations in pRS 316,
pPHY 3528	Atg1 with S_{677}A, S_{680}A, S_{683}A mutations in pRS 316,
pPHY 3560	Atg1 with S_{365}A mutation in pRS 316, made from pPHY 1115
pPHY 3562	Atg1 with S_{680}A mutation in pRS 316, made from pPHY 1115
pPHY 3564	Atg1 with S_{683}A mutation in pRS 316, made from pPHY 1115
pPHY 3566	Atg1 with S_{769}A mutation in pRS 316, made from pPHY 1115
pPHY 3568	Atg1 with S_{783}A mutation in pRS 316, made from pPHY 1115
pPHY 3570	Atg1 with S_{621}A mutation in pRS 316, subcloned from pPHY 2792 into pPHY 1115
pPHY 3572	Atg1 with S_{677}A mutation in pRS 316, subcloned from pPHY 2793 into pPHY 1115

Strains and growth media: Standard *E. coli* growth conditions and media were used throughout this study. The yeast rich growth medium, YPAD, consists of 1% yeast extract, 2% Bacto peptone, 500 mg/L adenine-HCl, and 2% glucose. The yeast YM glucose and SC glucose minimal growth media have been described (Kaiser *et al.* 1994). Growth media reagents were from DIFCO. The yeast strains used were PHY 2801 (TN125 *MATa ade2 his3 leu2 lys2 trp1 ura3 pho8::pho8Δ60*), PHY 2802 (YYK126 *MATa ade2 his3 lys2 trp1 ura3 pho8::pho8Δ60 atg1Δ::LEU2*), PHY 4167 (BY 4741 *MATa his3 leu2 met15 ura3 atg1Δ::KANMX),* PHY 4201 *(*BY 4741 *MATa his3 leu2 met15 ura3 atg13Δ::KANMX),* and PHY 4205 (BY 4741 *MATa his3 leu2 met15 ura3 atg17Δ::KANMX)* (Noda *et al.* 1995; Kamada *et al.* 2000; Glaever *et al.* 2002).

Mass Spectrometry analysis: Tandem MS/MS was performed on Atg1 protein in collaboration with Dr. Kay-Hooi Khoo in Academia Sinica, Taiwan. GST-tagged Atg1 received from Dr. Mike Synder was grown in SC with 2% raffinose until OD_{600} 0.6, and then induced with 3% galactose for 4 hours. The samples were run on SDS-gel, which was stained with G-250 commassie blue staining. The corresponding band of Atg1 was excised and sent to Taiwan. In brief, the protein sample was digested specifically at arginine and lysine by trypsin. The tryptic peptides were analyzed by MS and MS/MS approaches to determine the site of phosphorylation on Atg1. The composite of this analysis identified 15 phosphorylation sites with overall peptide coverage of 54% (Fig 5).

Subcloning: Previously made mutations within Atg1 in high-copy plasmid (pPHY 1249) were subcloned out of Atg1 by *PflMI* and *BglII* and ligated into pPHY 1115.

Gap repair to introduce a site-directed mutation: pPHY 1115 was linearized near amino acid 513 by a unique *PshAI* restriction digest. Standard PCR procedures were used. Reactions contained 200 μM dNTPs, 0.5 μM of each primer, 200 ng of template, 1x PCR reaction buffer and *Taq* polymerase. One of the PCR primers for each site engineered the missense mutation to code for alanine at the site, and any synonymous mutation needed to create a new restriction site for subsequent screening. Cycling parameters were 94˚C for 2 min, 25 cycles of 94˚C for 25s, 60˚C for 45s, and 72˚C for 8 min. The PCR products spanned the gap made by *PshAI*. 2.5 μg of the PCR product and 0.05 μg of linearized pPHY 1115 were transformed into PHY 2801 yeast cells. The cells were plated with Ura selection, and the resulting homologous recombined plasmid was extracted from yeast. The rescued plasmid was transformed into *E. coli* and restriction digestion specific to the mutation was used to screen for clones. Clones were purified by QIAGEN miniprep kit and DNA sequenced by the Ohio State University Plant-Microbe Genomics Facility. Further analysis of mutants was performed in PHY 2802 yeast cells.

Western blotting: Protein samples for Western blotting were prepared by centrifuging 10 ODs of cells (OD_{600} 0.6-0.8) and they were resuspended in 200 μl of 2x urea sample buffer (8M urea, 40mM Tris, 10% β-Mercaproethanol, 6% SDS). The cells were vortexed with glass beads for 5 min at 4˚C, and then heated for 5 min at 65˚C. The cell extracts were centrifuged at 12,000 × *g* for 2 min and the protein extract was used for subsequent Western blotting. The proteins were separated on SDS polyacrylamide gels, transferred to nitrocellulose membranes and the membranes were probed with the appropriate monoclonal primary (α-myc; Cell signaling) and secondary (α-mouse; GE Healthcare) antibodies. The Supersignal chemiluminescent substrate (Pierce) was used to detect the reactive bands.

Autophagy assays: The alkaline phosphatase (ALP) based assay measures the delivery and subsequent activation in the vacuole of an altered form of the Pho8 alkaline phosphatase, known as Pho8Δ60 (Noda *et al.* 1995). The increase in alkaline phosphatase activity that was observed following the period of starvation or rapamycin treatment is a direct measure of the autophagy activity present in those cells. For the assays, the cells were resuspended in 300 µl of the assay buffer (250 mM Tris-SO_4, pH 9.4, 10 mM $MgSO_4$, 10 µM $ZnSO_4$) and then vortexed with glass beads. The cell lysates were clarified by centrifugation at 10,000 × *g* for 5 min, and 20 µl of the resulting protein extract was added to a tube containing 480 µl of assay buffer and 1.25 mg/ml solution of *p*-nitrophenylphosphate. This reaction mix was incubated for 30 min at 35 °C, and the reaction was then terminated by the addition of 500 µl of 2 M glycine-NaOH, pH 11. The absorbance at 405 nm of the resulting solution was then measured (Klionsky and Emr 1989; Noda and Klionsky 2008). The protein concentrations in the cell extracts were determined with a bicinchoninic acid protein assay kit (Pierce). For the rapamycin experiments, autophagy assays were performed on cells that had been treated with 0.2 µg/ml rapamycin for 4 h at 30°C. The relative level of autophagy was indicated by the difference in alkaline phosphatase activity detected between extracts prepared from log phase and rapamycin-treated cells. The data presented represent the average of at least three independent experiments.

Chapter 3

Results

Aim I: Importance of phosphorylation within the activation loop

Atg1 kinase activity required phosphorylation at Thr 226 in the activation loop

A Western blot assay was used to determine Atg1 kinase activity *in vivo*. Kinase active Atg1 formed a second, slower migrating band (Atg1-P) under starvation conditions and rapamycin treatment. Inhibition of TOR signaling by the drug, rapamycin, has been shown to induce autophagy and mimic starvation conditions in yeast (Noda and Ohsumi 1998.) The Atg1-P band is lost upon treatment with phosphatase, therefore is generated by phosphorylation on Atg1 (Stephan *et al.* 2009). A single phosphorylation site at Ser-390 causes the shift to the upper band. In addition, the Atg1-P band is absent when required components for Atg1 kinase activity like Atg13 or Atg17 are absent (Fig 1). The activation loop phosphorylation is a highly conserved regulatory mechanism for certain kinases, and is required for a fully active kinase (Nolen *et al.* 2004). To assess the significance of phosphorylation in the activation loop of Atg1, we replaced the threonine at position 226 to alanine, a nonphosphorylatable amino acid ($Atg1^{T226A}$). This variant did not form a band in rapamycin conditions corresponding to Atg1-P (Fig. 2). This indicated that $Atg1^{T226A}$ lacks kinase activity, and that phosphorylation at T226 is important for the activation of Atg1 kinase.

T226A allele cannot induce autophagy

Since Atg1 kinase activity is required for the induction of autophagy, we wanted to test the kinase defective $Atg1^{T226A}$ for autophagy activity. For this purpose, we used an alkaline phosphatase (ALP) based assay. The ALP assay assesses the vacuolar delivery and processing of a truncated form of the Pho8 alkaline phosphatase, known as Pho8Δ60. This Pho8 variant lacks the targeting information necessary for its normal delivery to the vacuole

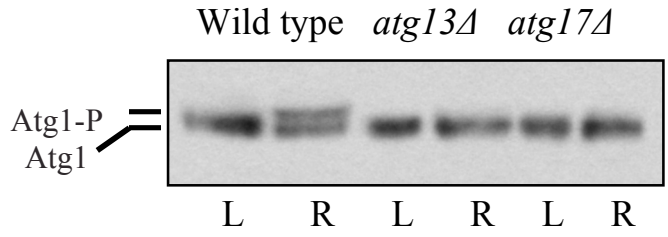
via the secretory pathway (Klionsky and Emr 1989; Noda *et al.* 1995). This altered protein can only be delivered to the vacuole by the autophagy pathway. Using this assay, Atg1^{T226A} showed no significant induction of autophagy and was comparable to the kinase defective strain (D211A) or *atg1Δ* strain (Fig 3). These data indicate that phosphorylation within the activation loop is necessary for the induction of autophagy.

T226E allele bypasses the need of Atg13 and Atg17 in autophosphorylation

Phosphorylation at T226 is important for Atg1 kinase activity, so we mutated T226 to either a glutamic acid or aspartic acid, phosphomimics. Atg1^{T226E} forms Atg1-P in rapamycin treated cells as well as log phase cells (Fig. 4). Atg1^{T226E} is a constituently active kinase, and we wanted to determine if Atg13 and Atg17 are still required for Atg1 kinase activity for this variant. The Atg1-P band formed in both *atg13Δ* and *atg17Δ* cells expressing the Atg1^{T226E} variant demonstrating that the requirement of Atg13 and Atg17 was bypassed (Fig 4). Together, these data suggest that phosphorylation at T226 is important for the activation of Atg1 kinase, and Atg13 and Atg17 are important for the phosphorylation at the activation loop.

Figure 1

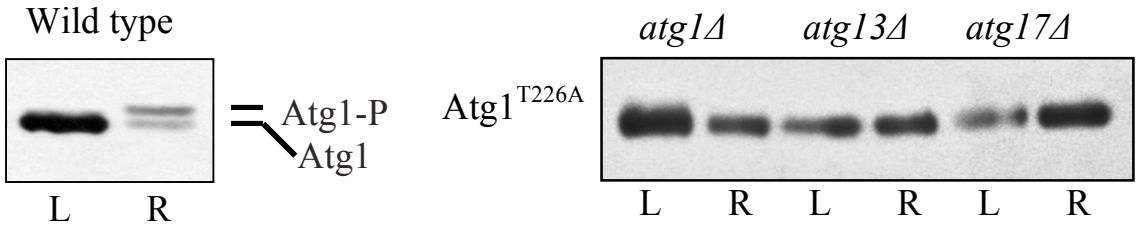
Atg13 and Atg17 a requirement for Atg1 kinase activity *in vivo*



The loss of Atg1 autophosphorylation *in vivo* occurred in the absence of Atg13 or Atg17. Western blots were performed with extracts prepared from either log phase (L) or rapamycin-treated (R) cells with Atg1 protein.

Figure 2

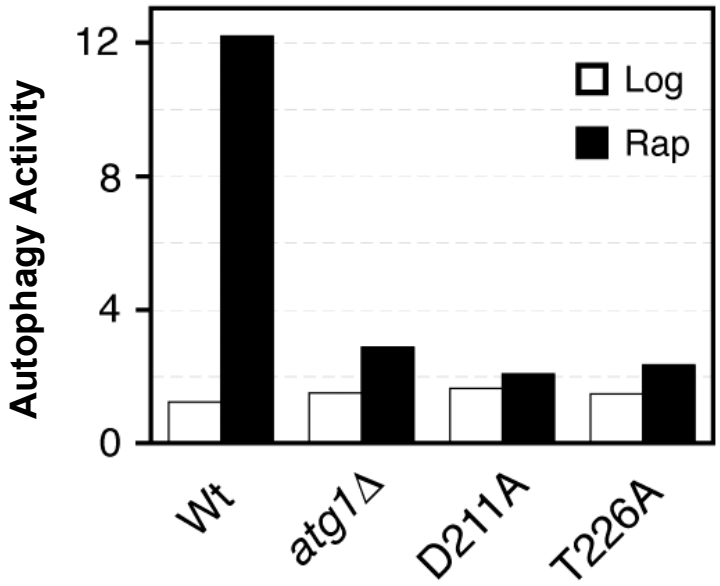
Atg1^{T226A} variant was defective for Atg1 autophosphorylation *in vivo*



The presence of the T226A alteration resulted in a loss of Atg1 autophosphorylation *in vivo*. Western blots were performed with extracts prepared from either log phase (L) or rapamycin-treated (R) cells with the indicated Atg1 proteins.

Figure 3

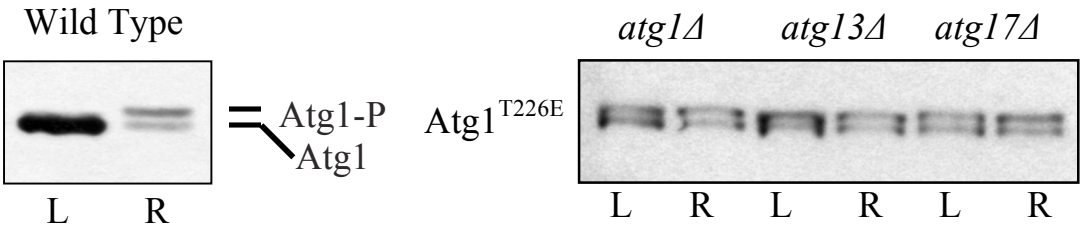
Atg1^{T226A} variant was defective for autophagy.



Autophagy activity was assessed with the ALP Pho8Δ60-based assay using an *atg1*Δ strain expressing the indicated Atg1 proteins. The *atg1*Δ control strain contained a vector plasmid. Extracts were prepared from cells that had been treated with 200 ng/ml rapamycin for either 0 (*Log*) or 4 hrs (*Rap*). The data shown are the average of two independent experiments.

Figure 4

Atg1^{T226E} variant bypassed the Atg13 and Atg17 requirement



The requirement of Atg13 and Atg17 in Atg1 autophosphorylation is bypassed with the T226E altered Atg1 allele. Western blots were performed with extracts prepared from either log phase (L) or rapamycin-treated (R) cells with the indicated Atg1 proteins.

Aim II: Examine the significance of other phosphorylation sites on Atg1

Elevated Atg1 kinase activity with S683A single and S677,680,683A triple mutants

A mass spectrometry analysis identified 15 phosphorylation sites on Atg1 with an overall coverage of 54% (Fig 5). These sites represent a subset of the possible phosphorylation events in Atg1. We individually mutated seven serine residues that were determined by MS to be phosphorylation sites (a list of the positions can be found in Table 2). Due to the proximity of certain sites, we constructed a triple mutant, $Atg1^{S677,680,683A}$. Three phosphorylation sites, which had similar adjacent sequences, were combined to create a second triple mutant, $Atg1^{S621,677,683A}$. To evaluate how these phosphorylation sites affect Atg1 kinase activity, we analyzed each of the mutants using the Western blot assay described previously. Most of the single serine-to-alanine mutants were similar to wild type Atg1 except for $Atg1^{S683A}$. $Atg1^{S683A}$ and a triplet mutant $Atg1^{S677,680,683A}$ showed a stronger signal in the upper band, Atg1-P band, after rapamycin treatment (Fig 6). This modest increase of the Atg1-P band for the 2 mutants could demonstrate an increase in Atg1 kinase activity under rapamycin treatment conditions.

Induction of autophagy was modestly decreased by a subset of mutants

We analyzed the serine-to-alanine mutants for the ability to induce autophagy. We used the alkaline phosphatase assay described above to investigate each mutant. A subset of mutants showed a 30-40% decrease in autophagy activity including the triplet mutant $Atg1^{S677,680,683A}$ and a number of single serine-to-alanine mutations including at amino acid positions 365, 677, 769, and 783 (Fig 7). All other variants of Atg1 analyzed displayed similar induction of autophagy as wild type Atg1. None of the mutants showed a significant change in autophagy activity compared to wild type under normal growth conditions (data not shown). The modest decrease in the induction of autophagy of several of the mutants

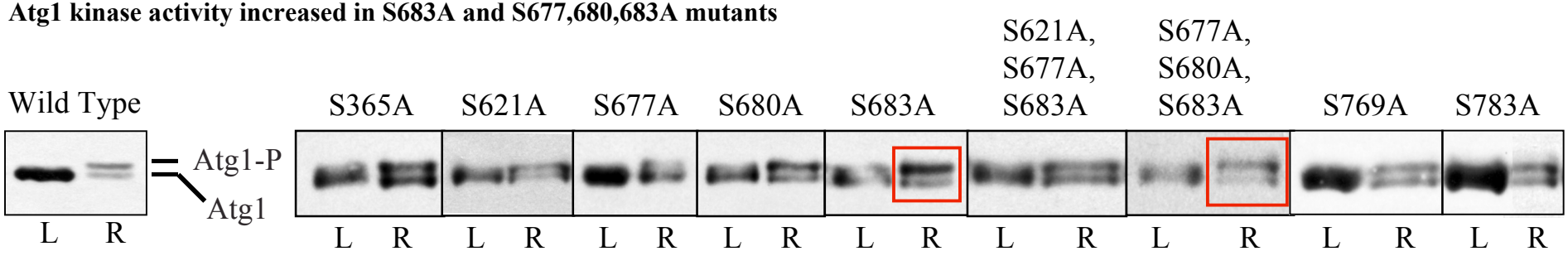
studied indicates the possible need of multiple phosphorylations on Atg1 to affect the autophagy activity.

Figure 5

Fifteen phosphorylation sites were found with peptides analyzed representing 54% of the protein.

Figure 6

Atg1 kinase activity increased in S683A and S677,680,683A mutants

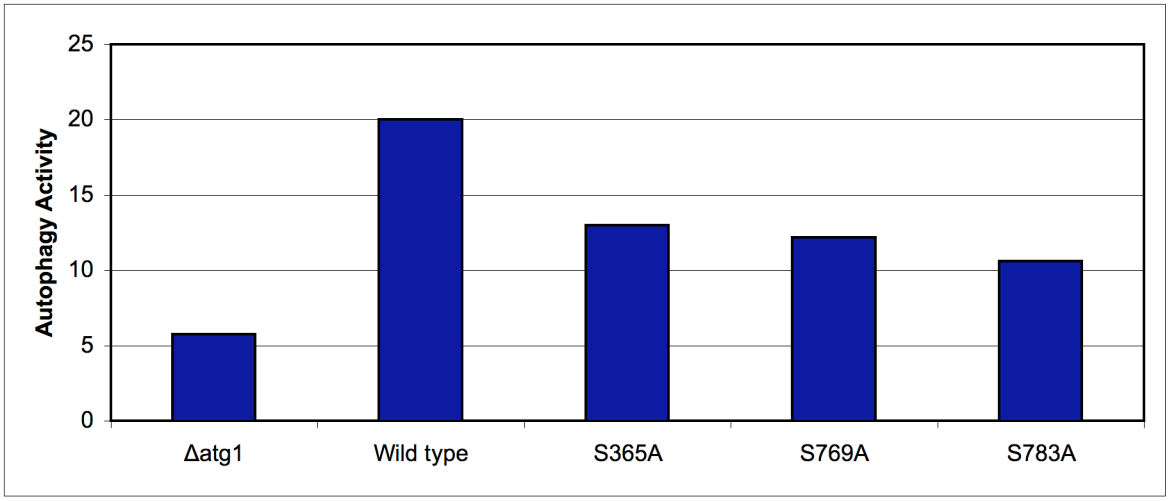


The increased Atg1-P band in both Atg1S683A, and Atg1S677,680,683A indicates an increase in Atg1 kinase activity. Western blots were performed with extracts prepared from either log phase (L) or rapamycin-treated (R) cells with the indicated Atg1 protein variants.

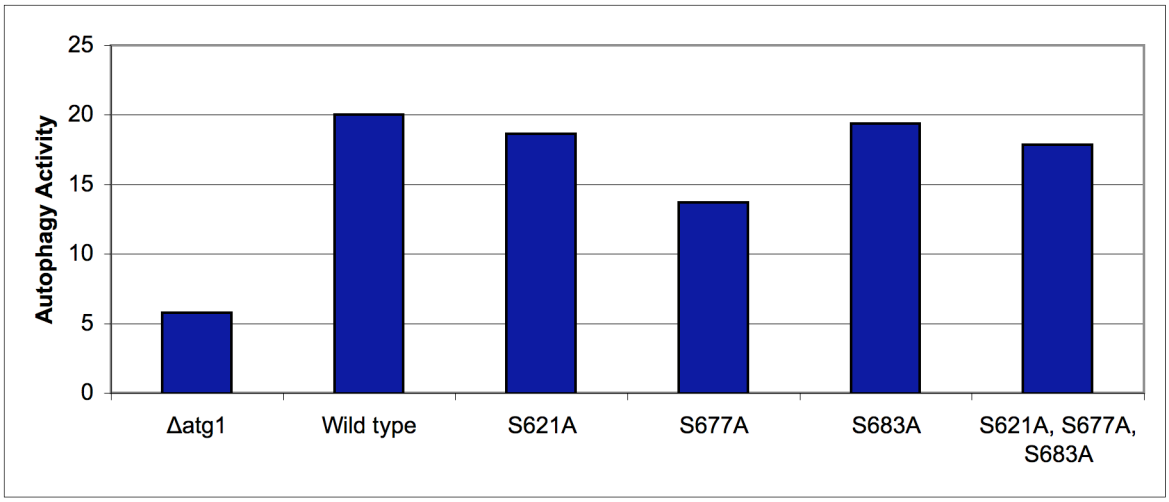
Figure 7

Autophagy activity is modestly decreased in a subset of mutants

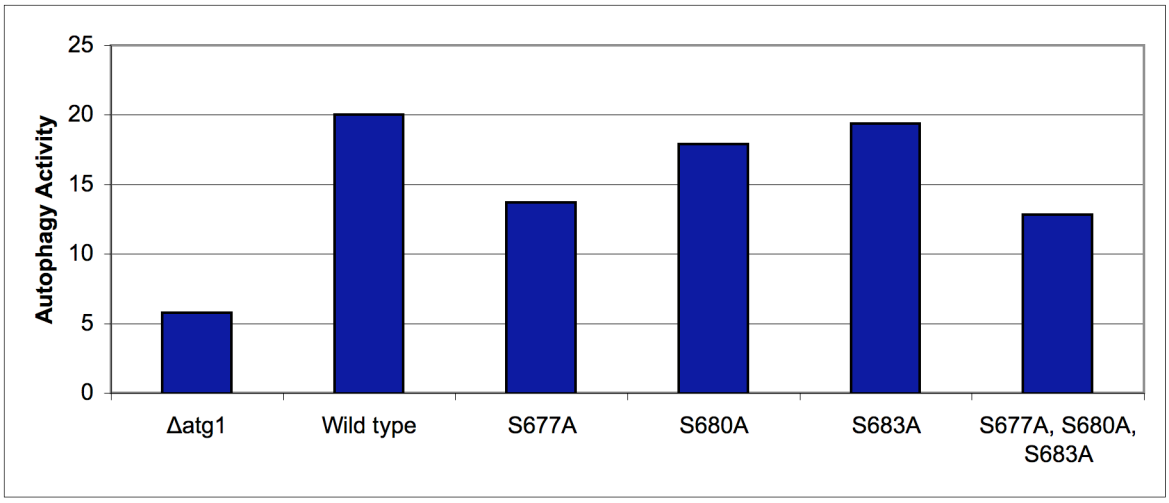
A



B



C



Autophagy activity was assessed with the ALP Pho8Δ60-based assay using an *atg1*Δ strain expressing the indicated Atg1 proteins. The *atg1*Δ control strain contained a vector plasmid. Extracts were prepared from cells that had been treated with 200ng/ml rapamycin for 4 h. The data shown are the average of at least three independent experiments normalized to wild type.

Table 2

Summary of Results		
Altered sites of phosphorylation	Atg1 kinase activity	Autophagy levels (% of wild type)
S365A	WT	**65%**
S621A	WT	100%
S677A	WT	**68%**
S680A	WT	100%
S683A	**Increased Atg1-P band**	100%
S769A	WT	**61%**
S783A	WT	**53%**
S621A, S677A, S683A	WT	100%
S677A, S680A, S683A	**Increased Atg1-P band**	**64%**

Chapter 4

Discussion

Autophagy has been implicated in a wide variety of diseases and has been suggested as a potential therapeutic target for many of these ailments (Levine and Kroemer 2008). For the purpose of utilizing autophagy as a therapeutic tool, it is vital that we develop a thorough understanding of how this pathway is regulated and controlled. Atg1 is a key regulator of autophagy and therefore a key target for manipulation in the pathway. The work here identifies a specific phosphorylation event within the activation loop of Atg1 as a potential point of control for autophagy in *S. cerevisiae,* and reveals more possible regulation events on Atg1.

The activation loop is a conserved domain within the kinase domain of many kinases. Phosphorylation within this loop permits appropriate binding of substrate with the kinase domain and/or facilitates the phosphorylation reaction (Johnson *et al*. 1996; Nolen *et al*. 2004). Phosphorylation within the activation loop of many protein kinases is required for their full activity and this can be an essential event for their activation (Nolen *et al.* 2004). We found that the ability to phosphorylate within the activation loop is necessary for Atg1 kinase activity. The presence of two previously-identified regulators of Atg1 kinase activity, Atg13 and Atg17, was required for this phosphorylation event. Interestingly, Atg1 no longer required the presence of Atg13 or Atg17 for autophosphorylation *in vivo* when T226 was replaced with a phosphomimetic, such as glutamic acid. Bypassing the necessity for these regulators suggests that one of the functions of the Atg13 and Atg17 proteins during autophagy is facilitating Atg1 autophosphorylation within the activation loop.

The MS analysis of Atg1 uncovered numerous potential phosphorylation sites within the protein. My work here found that no single phosphorylation site tested was absolutely necessary for Atg1 kinase activity or autophagy. However, many showed modest changes in either Atg1 kinase activity or autophagy. Single mutations may cause a conformational change within the C-terminal region of Atg1 and confer a slight decrease in binding affinity with partners for the necessary complex to induce autophagy. A subset of single phosphorylations analyzed was found to promote autophagy and dephosphorylation at Ser-683 increases Atg1 kinase activity. The S621,677,683A triplet allele showed normal induction of autophagy demonstrating that the S621A mutation suppresses the defect in autophagy seen with S677A allele. This leads us to believe that the regulation of Atg1 is complex and might require multiple phosphorylation events. The phosphorylations could work cooperatively, and many changes must be made to cause a complete change in autophagy or Atg1 kinase activity. Interestingly, one of the triple mutants, $Atg1^{S677,680,683A}$, showed an increase in autophosphorylation, but impaired autophagy activity. Additional analysis of these phosphorylation sites along Atg1 is needed to clarify the roles of these events and how they coordinate the activation of Atg1 with autophagy. In view of the fact that Atg1 and its regulators have been conserved through evolution, further analysis in budding yeast could help us gain valuable insights into the regulation of this degradative process in higher eukaryotes.

References

Budovskaya, Y.V., J. S. Stephan, F. Reggiori, D.J. Klionsky and P.K. Herman, 2004 The Ras/cAMP-dependent protein kinase signaling pathway regulates an early step of the autophagy process in Saccharomyces cerevisiae. J Biol Chem **279:** 20663-20671.

Budovskaya, Y., Stephan, J., Deminoff, S. and Herman, P. 2005. An evolutionary proteomics approach of the cAMP-dependant protein kinase. PNAS 102, 13933-13938.

Chen N., and V. Karantza-Wadsworth, 2009 Role and regulation of autophagy in cancer. Biochim. Biophs. Acta. 1793(9):1516-23.

Giaever, G., A.M. Chu, L. Ni, C. Connelly, L. Riles *et al.*, 2002 Functional profiling of the *Saccharomyces cerevisiae* genome. Nature 418: 387-391.

He, C., and D. J. Klionsky, 2009 Regulation Mechanisms and Signaling Pathways of Autophagy. Annu Rev Genet.

Johnson, L. N., M. E. Noble and D. J. Owen, 1996 Active and inactive protein kinases: structural basis for regulation. Cell 85: 149-158.

Johnson, L. N., and M. O'Reilly, 1996 Control by phosphorylation. Curr Opin Struct Biol 6:762-769.

Kaiser, C., S. Michaelis and A. Mitchell, 1994 *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Kamada, Y., Funakoshi, T., Shintani, T., Nagano, K. Ohsumi, M. and Ohsumi Y. (2000). Tor-mediated Induction of Autophag Via an Apg1 Protein Kinase Complex. Cell Biology 150, 1507-1513.

Kamada, Y., K. Yoshino, C. Kondo, T. Kawamata, N. Oshiro *et al.*, 2010 Tor directly controls the Atg1 kinase complex to regulate autophagy. Mol Cell Biol 30: 1049-1058.

Klionsky, D.J., and S.D. Emr, 1989 Membrane protein sorting: biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase. EMBO J 8: 2241-2250.

Levine, B., and G. Kroemer, 2008. Autophagy in the Pathogenesis of Disease. Cell 132, 27-42.

Meijer, W. H., I. J. Van der Klei, M. Veenhuis and J. A. Kiel, 2007 *ATG* genes involved in non-selective autophagy are conserved from yeast to man, but the selective Cvt and pexophagy pathways also require organism-specific genes. Autophagy 3: 106-116.

Noda, T., and D.J. Klionsky, 2008 The quantitative Pho8Delta60 assay of nonspecific autophagy. Methods Enzymol 451: 33-42.

Noda, T., A. Matsuura, Y. Wada, and Y. Ohsumi, 1995 Novel system for monitoring autophagy in the yeast Saccharomyces cerevisiae. Biochem Biophys Res Commun 210: 126-132.

Noda, T., and Y. Ohsumi, 1998 Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. J Biol Chem 273: 3963-3966.

Nolen, B., S. Taylor, and G. Ghosh, 2004 Regulation of protein kinases; controlling activity through activation segment conformation. Mol Cell 15: 661-675.

Stephan, J.S., Y.Y. Yeh, V. Ramachandran, S.J. Deminoff and P.K. Herman, 2009 The Tor and PKA signaling pathways independently target the Atg1/Atg13 protein kinase complex to control autophagy. Proc Natl Acad Sci U S A 106: 17049-17054.

Suzuki, K., and Y. Ohsumi, 2007 Molecular machinery of autophagosome formation in yeast, *Saccharomyces cerevisiae*. FEBS Lett 581: 2156-2161.

Tsukada, M., and Y. Ohsumi, 1993 Isolation and characterization of autophagy-defective mutants of *Saccharomyces cerevisiae*. FEBS Lett 333: 169-174.

Xie, Z., and D. J. Klionsky, 2007 Autophagosome formation: core machinery and adaptations. Nat Cell Biol 9: 1102-1109.

Yeh, Y.Y., K. Wrasman, and PK Herman, 2010, Autophosphorylation Within the Atg1 Activation Loop is Required for Both Kinase Activity and the Induction of Autophagy in Saccharomyces cerevisiae. Genetics.